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Expression of extracellular glutathione peroxidase type 5 (GPX5) in the rat male reproductive tract

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The mammalian epididymis is the site of expression and secretion of an abundant, tissue-specific, androgen-regulated, selenium-independent, glutathione peroxidase isoenzyme (GPX5), which has been proposed to play a role in protecting the membranes of spermatozoa from the damaging effects of lipid peroxidation and/or preventing premature acrosome reaction. Using a combination of reverse transcription–polymerase chain reaction, Northern blot analysis and Western blotting, we now describe in detail the developmental expression of GPX5 transcripts and protein in the rat epididymis and characterize the association of rat GPX5 with the sperm plasma membrane.

Key words: epididymis/glutathione peroxidase/lipid peroxidation/reactive oxygen species/spermatozoa

Introduction

The membranes of mammalian spermatozoa are rich in polyunsaturated fatty acids, making them particularly sensitive to the deleterious effects of lipid peroxidation, which may result in rapid and irreversible loss of motility and a low level of fertilizing capacity (recently reviewed by Storey, 1997). A membrane that has sustained free radical-mediated lipid peroxidation will exhibit changes in membrane permeability and altered transmembrane ionic gradients. The fluidity of the membrane is affected and lipid–protein interactions may be modified. Cleavage of the carbon bonds during the rounds of lipid peroxidation results in the formation of aldehydic products such as cytotoxic alkanals and alkenals as well as alkanes and cyclic endoperoxides. These in turn are toxic compounds which are capable of reacting with the amino and thiol groups of proteins, thereby disrupting normal functions and affecting cell viability.

Within the male reproductive tract, glutathione peroxidase (GPX) activity is proposed to play an important role in the inactivation of hydrogen peroxide and organic hydroperoxides. Glutathione peroxidase activity in mammalian cells is exhibited by multiple isoenzymes, four of which are selenium-containing enzymes.

The classical cellular GPX (GPX1) is a tetrameric protein which detoxifies both hydrogen peroxide and soluble organic hydroperoxides, using reduced glutathione (GSH) as reducing equivalents. This enzyme plays a well-established, major role in the prevention of free radical/reactive oxygen species (ROS) damage within cells (Flohe *et al.*, 1973).

Phospholipid hydroperoxide glutathione peroxidase (GPX4) is a monomeric, glutathione-dependent, cytosolic enzyme, capable of catalysing the reduction of phospholipid hydroperoxide, cholesterol hydroperoxide and linoleic acid hydroperoxide (Thomas *et al.*, 1990). This enzyme is especially abundant in the rat testis, reproductive tissues and endocrine tissues (Roveri *et al.*, 1994). Interestingly, these same tissues have only low concentrations of GPX1 (Roveri *et al.*, 1992). The monomeric structure opens up the accessibility of the active site and this may account for the ability of GPX4 to act on lipid peroxides within the lipid membrane. Although GPX4 lacks the charged residues implicated in binding GSH, GSH still appears to be the most efficient electron donor found to date (Ursini *et al.*, 1985). Recently, Godeas *et al.* (1997) have proposed an additional role for GPX4 in the maturation of spermatozoa *via* the metabolism of hydroperoxides and sperm thiol oxidation.

Plasma glutathione peroxidase (GPX3) is a tetrameric glycoprotein which is secreted into the plasma, milk and lung (Takahashi *et al.*, 1987; Avissar *et al.*, 1994). Whilst primarily a secreted protein, high concentrations are associated with the cells of the placenta (Avissar *et al.*, 1994). GPX3 transcripts have also been detected in human (A.C.F.Perry and L.Hall, unpublished data) and mouse (Schwaab *et al.*, 1995) epididymis. GPX3 is capable of reducing soluble phospholipid peroxides as well as hydrogen peroxide and alkyl hydroperoxides (Yamamoto and Takahashi, 1993). It is unlikely that reduced glutathione is the physiological substrate used in view of its low concentration within the plasma. Bjornsted *et al.* (1994) have suggested two alternative electron donors for GPX3: the thioredoxin and glutaredoxin systems. Both systems proved to be capable and efficient electron donors for plasma GPX and both have a recycling pathway available within the plasma environment.

Finally, gastrointestinal GPX (GPX2) is expressed in the liver, as well as the gastrointestinal tract (Akasaka *et al.*, 1990; Chu *et al.*, 1993). GPX2 can utilize glutathione as its electron donor and within the intestinal tract there is reduced glutathione available in millimolar concentrations, sufficient to ensure efficient catalysis.

Each of the above GPX isoenzymes possesses a seleno-

cysteine residue at its active site, which acts via redox reactions to bring about catalysis. The lower pK and higher nucleophilicity of selenium compared to sulphur make for a more reactive redox centre. However, selenium-independent GPX isoenzymes have also been identified. These enzymes share a high degree of sequence identity to the selenium-containing isoenzymes, but possess a normal cysteine residue in place of the active site selenocysteine residue.

The first cloned cDNA for a selenocysteine-independent, secreted GPX was obtained using mouse epididymal RNA as template (Ghyselinck and Dufaure, 1990; Ghyselinck *et al.*, 1991). This androgen-regulated mRNA was localized exclusively to the principal cells of the caput epididymis and a developmental profile revealed transcripts at 10 days post-partum, rising steadily in the run up to puberty (Faure *et al.*, 1991; Lefrancois *et al.*, 1993). As well as being present in the epididymal lumen, immunolocalization studies have established that mouse epididymal GPX (GPX5) is bound to the head region of spermatozoa from the caput, corpus and cauda epididymis, and on spermatozoa ejaculated into the female and retrieved from the uterine horn (Vernet *et al.*, 1997). Recently, polyoma enhancer activator protein (PEA3), a member of the *Ets* oncogene family, has been proposed to play a role in the tissue-specific regulation of mouse GPX5 expression (Drevet *et al.*, 1998).

Full-length cDNA encoding the rat and macaque (*Macaca fascicularis*) orthologues of mouse GPX5 were obtained by Perry *et al.* (1992). Using these cloned cDNAs as probes for Northern blotting experiments, GPX5 transcripts were localized exclusively to the epididymis in both the rat and macaque (Perry *et al.*, 1992). Castration, and subsequent testosterone treatment of castrated rats, demonstrated the androgen dependence of GPX5 transcript concentrations (Perry *et al.*, 1992), in agreement with work done in the mouse (Ghyselinck and Dufaure, 1990; Lefrancois *et al.*, 1993).

Mammalian GPX5 transcripts encode a protein with a predicted relative molecular mass of ~24–25 kDa. These transcripts lack the UGA codon that permits insertion of the selenocysteine residue, instead using a normal UGY cysteine codon.

Using a combination of reverse transcription–polymerase chain reaction (RT–PCR), Northern blot analyses and Western blotting, we now expand our preliminary studies on the developmental expression of GPX5 transcripts and protein in the rat epididymis and further characterize the association of rat GPX5 with the sperm plasma membrane.

Materials and methods

Animals

Adult male Wistar rats (250–300 g) were housed under controlled temperature (22°C) and light (14 h/day). Food and water were supplied *ad libitum*. Animals were killed as directed by Schedule 1 of the Animals (Scientific Procedures) Act 1986 (UK).

Expression of a recombinant maltose binding protein (MBP)–GPX5 fusion protein and generation of polyclonal antisera

Synthetic oligonucleotide primers (5′-CTT GGA TCC GAA TTC ATG ACC CCC AGG CTG GAA AAG AT-3′ and 5′-CTG CAG GAT CCT AAG CTT ATA TGG TTT TGA ATT GAT T-3′) were used to amplify the mature coding region of rat GPX5 by PCR, using the corresponding cloned, full-length cDNA as the template (Perry *et al.*, 1992). The resulting PCR product was cloned into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA, USA), and expressed in *Escherichia coli*. The fusion protein was affinity-purified on an amylose–agarose resin column (New England Biolabs) and polyclonal antisera (designated J5 and J6) were raised in two New Zealand White rabbits, as described previously (Frayne *et al.*, 1998a).

Factor Xa protease cleavage of the recombinant rat GPX5 fusion protein

Purified MBP–GPX5 fusion protein (20–50 μg) was cleaved at room temperature by incubation with Factor Xa protease (New England Biolabs) in 20 mM Tris–HCl, pH 7.6, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM sodium azide over a time course of 1–24 h. Products were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent Western blot analysis as described below.

Western blot analysis of rat GPX5

Rat tissue protein extracts (testis, caput and cauda epididymis, kidney, liver and placenta) were prepared by homogenization of tissues (~100 g) in 4 volumes of phosphate buffered-saline (PBS; 154 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.2) containing 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (ICN Biomedicals Ltd, Thame, UK), followed by incubation on ice in the same buffer containing 1% (v/v) Triton X-100 (Sigma Chemical Co., Poole, UK). Homogenates were then centrifuged at 10 000 *g* and supernatants stored at −20°C until required.

Rat caput and cauda epididymidal spermatozoa were isolated as described by Jones *et al.* (1990) and used to prepare plasma membrane-enriched fractions by detergent extraction with 1% (v/v) Triton X-100 and vortex mixing (Jones, 1986). 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride was included in the preparations.

Deoxycholate sperm membrane extracts were prepared by mincing six rat epididymides (caput or cauda as appropriate) in PBS and dispersing spermatozoa by gentle shaking. The supernatant was then decanted into a microcentrifuge tube, centrifuged at 13 000 *g* for 10 min and the resulting supernatant (epididymidal plasma) retained for analysis. The pellet after centrifugation was resuspended in 5 volumes of 0.4% (w/v) sodium deoxycholate in PBS containing 50 mM Tris, pH 8.3. Following re-centrifugation at 13 000 *g* for 10 min, the supernatant (sperm deoxycholate extract) was removed and stored at −20°C until required. Protein concentration was determined using the method of Bradford (1976).

Proteins (100μg/lane) were separated by SDS–PAGE under reducing conditions on 12% (w/v) polyacrylamide gels, and then electroblotted onto polyvinylidene difluoride membranes (PVDF, PolyScreen™; NEN Life Science Products, Brussels, Belgium). Blots were first probed with anti-rat GPX5 polyclonal antiserum for 1 h, washed in PBS and then incubated with horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Dako Ltd, Bucks, UK) for 1 h. Detection of bound antibodies was achieved by enhanced chemiluminescence (Amersham ECL™; Amersham International, Amersham, UK) and exposure of membranes to Hyperfilm™ (Amersham). When required, the membranes were stripped by boiling in 0.1 M HCl for 30 min prior to re-probing.

Extraction of rat sperm peripheral membrane proteins by high salt or high pH

Rat cauda spermatozoa were washed by gentle centrifugation (300 *g*) in modified Biggers–Whitten–Whittingham medium (BWW;

Aitken, 1983) to remove any epididymal fluid and were resuspended at a concentration of 1×10^7 spermatozoa/ml, in either 0.5 M KCl, pH 7.4, or 0.1 M $NaHCO_3$, pH 11.5. The spermatozoa were incubated at room temperature for 30 min, before being pelleted by centrifugation at 1000 *g* for 10 min. Pellets were extracted with 1% (v/v) Nonidet P40 in PBS, pH 7.4, at 4°C for 45 min, centrifuged at 100 000 *g* for 1 h at 4°C, and the supernatant was dialysed for 1 h against 6.25 mM Tris–HCl, pH 7.4, containing 0.2% SDS.

Preparation of rat sperm smears and detection of GPX5 by indirect immunofluorescence

Spermatozoa (from rat testis, caput or cauda epididymidis, or vas deferens) were separated from their surrounding fluid by repeated 1 ml washes with PBS followed by low speed centrifugation at 300 *g* and resuspension of the pellet in PBS. Aliquots (5 µl) were placed on a clean glass slide, smeared with the edge of a second slide and air-dried at room temperature.

Sperm smears were rehydrated in PBS before being blocked with 5% normal swine serum in PBS for 1 h at room temperature. Following a 5 min wash in PBS, the smears were incubated with anti-rat GPX5 polyclonal antiserum (diluted 1:200 in PBS) for 1 h at room temperature. This was followed by three successive washes in PBS followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit second antibody (diluted 1:50 in PBS) at room temperature for 1 h. After three further washes in PBS, sections were mounted using fluorescent mountant (Dako Ltd, High Wycombe, UK) and viewed under fluorescence light with a Leica microscope.

Northern blot analysis of rat GPX5 transcripts

Adult rat epididymides were dissected into their distinct regions (proximal caput, distal caput, corpus, proximal cauda and distal cauda) based on surface morphology, snap-frozen in liquid nitrogen and stored at –80°C until required. Total RNA was extracted from these epididymal regions using an SDS/proteinase K-based method as described previously (Girotti *et al.*, 1992). RNA samples were then fractionated on a 1.2% (w/v) agarose gel containing 50% formaldehyde, blotted onto a Hybond™-N nylon filter and probed with a ^{32}P-labelled, rat GPX5 cloned cDNA (Perry *et al.*, 1992) as described by Perry *et al.* (1995).

Detection of rat GPX5 transcripts by RT–PCR

Total rat epididymides were removed from juvenile rats at 13, 19, 23, 32 and 44 days post-partum, as well as from adult rats, and snap-frozen in liquid nitrogen. Total RNA was then extracted (Frayne *et al.*, 1997) from 50–100 mg of tissue using TRIzol™ reagent (Gibco BRL, Paisley, UK). In addition, the TRIzol™ organic phase, which contains extracted proteins, was retained for Western blot analysis. Single-stranded cDNA was synthesized from aliquots of the RNA using oligo $(dT)_{12-18}$ as primer and Expand™ reverse transcriptase (Boehringer Mannheim, Lewes, UK). This cDNA was then used in PCR reactions using rat GPX5-specific oligonucleotide primers (5′-TTC CAG CGG ATG TCA TGG AC-3′ and 5′-GCT ACC TAT TGT GGT CTG AC-3′). PCR parameters were: 94°C for 1.5 min, cool to 58°C over 1.5 min, hold at 58°C for 2 min, 72°C for 2 min, for 30 cycles; using Expand™ High Fidelity PCR System (Boehringer Mannheim). Positive control reactions, using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5′-GAA CGG GAA GCT CAC TGG CAT G-3′ and 5′-GTC CAC CAC CCT GTT GCT GTA G-3′) were performed in order to confirm the integrity of the synthesized cDNA and correct for differences in RNA, and hence cDNA, yields between the different tissue samples.

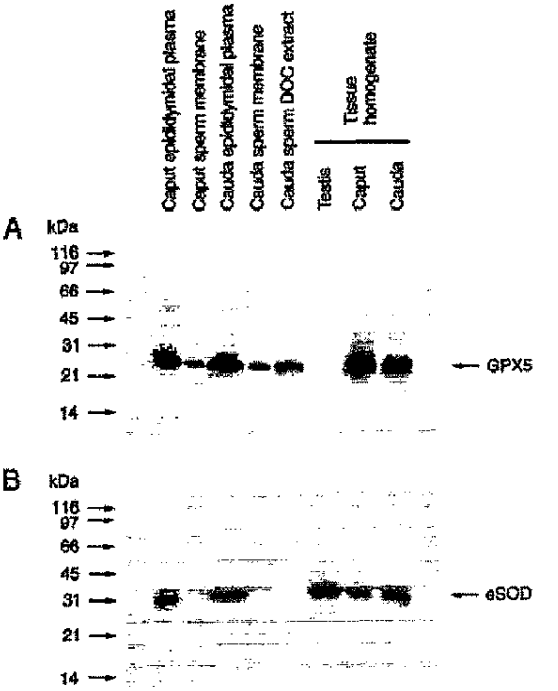

Figure 1. Western blot analysis of glutathione peroxidase isoenzyme (GPX5) in rat epididymis and testis. Tissue homogenates, membrane extracts and epididymal plasma (100 µg protein/lane) were probed with either (**A**) an anti-GPX5 polyclonal antiserum, or (**B**) an anti extracellular superoxide dismutase (eSOD) polyclonal antiserum. DOC = sodium deoxycholate.

Results

Identification of GPX5 in rat epididymal protein extracts by Western blotting

Polyclonal antisera (J5 and J6) raised in rabbits against a rat GPX5 recombinant fusion protein (see Materials and methods) reacted well with the corresponding, bacterially-expressed, GPX5 fusion protein and detected the expected 24 kDa GPX5 component following cleavage of the fusion protein with Factor Xa (data not shown). A band of 24 kDa was also detected by immune sera on Western blots (Figure 1A) of rat epididymal tissue homogenates, rat caput and cauda epididymidal plasma, rat caput and cauda sperm membrane preparations and a detergent (deoxycholate) extract of cauda spermatozoa, suggesting the association of GPX5 with sperm membranes, as well as its presence in epididymal fluids. Corresponding pre-immune sera (pre-immunization bleeds from the same animals) gave no detectable bands under the same conditions. The purity of sperm membrane fractions and the specific association of GPX5 with these membranes was further demonstrated by probing a parallel blot with a rat extracellular superoxide dismutase (eSOD) antiserum (Figure 1B). As expected, no eSOD band was detected in the lanes containing membrane fractions (eSOD is not membrane-associated), only in the tissue homogenate and epididymidal

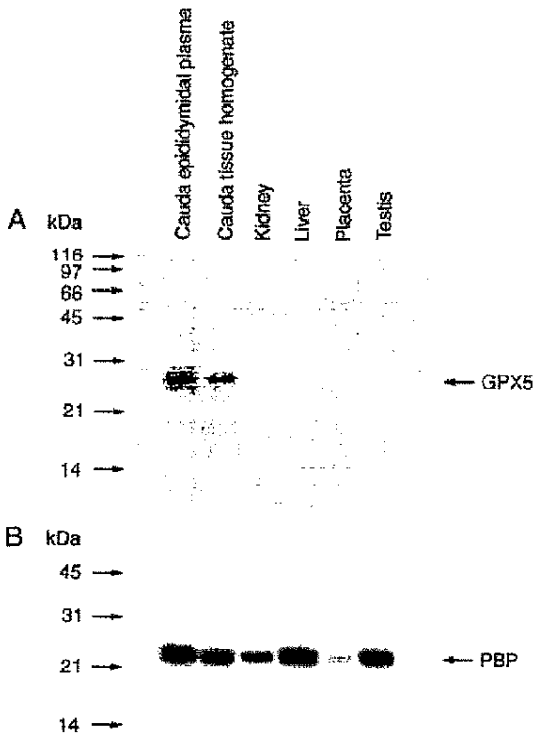

Figure 2. Western blot analysis of glutathione peroxidase isoenzyme (GPX5) in rat tissue homogenates. Tissue homogenates (cauda epididymidis, kidney, liver, placenta and testis) and cauda epididymidal plasma (100 µg protein/lane) were probed with an anti-GPX5 polyclonal antiserum (**A**). The blot was then stripped and re-probed using using an anti-phosphatidylethanolamine-binding-protein polyclonal antiserum (**B**) to confirm the integrity of the samples.

plasma samples (eSOD is secreted by the epididymis; see Perry *et al.*, 1993).

In view of the range of GPX isoforms, it was important to establish the specificity of the GPX5 antisera. In particular, for meaningful experiments to be carried out on the expression and localization of GPX5, potential cross-reactivity with the closely related plasma GPX3 isoenzyme had to be examined. Western blots were therefore carried out with protein extracts from a range of rat tissues, some known to express high concentrations of GPX3 (e.g. placenta), but no significant cross-reaction was observed (see Figure 2A). To confirm equal lane loading and the integrity of the protein samples, the Western blot was stripped and reprobed (Figure 2B) with an antiserum against constitutively expressed rat phosphatidylethanolamine binding protein (PBP; results not shown) (Frayne *et al.*, 1998b),

Association of GPX5 with rat sperm membranes

GPX5 protein has been localized to the sperm acrosomal cap in the mouse (Jimenez *et al.*, 1990; Vernet *et al.*, 1997) and more recently in the pig (Okamura *et al.*, 1997), where it is presumed to represent a peripheral membrane protein. There is also a pool of free GPX5 in mouse epididymal fluid, as expected for an epididymal secreted protein.

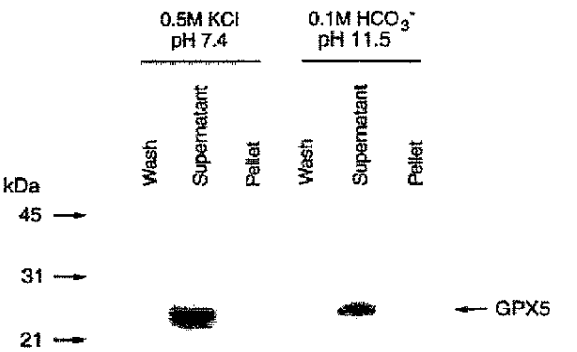

Figure 3. Release of glutathione peroxidase isoenzyme (GPX5) from rat sperm membranes using high salt (0.5 M KCl, pH 7.4) or high pH (0.1 M $NaHCO_3$, pH 11.5) and then centrifuged as described in the Materials and methods section. Membrane-bound GPX5 (in the sperm pellet) and released GPX5 (in the supernatant) were detected by Western blot analysis.

Initial examination of rat GPX5 showed that extensive washes with PBS and/or BWW did not remove the protein from the sperm surface, whereas incubation with 1% Nonidet P-40 was effective in its removal (data not shown). However, exposure of washed rat spermatozoa to high concentrations of salt (0.5 M KCl, pH7.4) or high pH (0.1 M Na_2CO_3, pH 11.5) both resulted in the removal of GPX5 from the membrane (Figure 3), indicating a peripheral (rather than integral) membrane protein. Indeed the secretory nature of GPX5 and the absence of a candidate transmembrane domain would argue against it being an integral membrane protein.

Localization of GPX5 on rat spermatozoa by indirect immunofluorescence

The presence of GPX5 on rat spermatozoa, indicated by Western blot analysis, was confirmed by indirect immunofluorescence using the anti-GPX5 polyclonal antiserum. In preliminary experiments, sperm smears were examined using this antiserum at various antiserum concentrations and the optimum dilution was found to be 1:200. Subsequent sperm smears incubated with immune serum at this dilution, followed by FITC-conjugated second antibody, consistently produced a distinct crescent-shaped area of fluorescence over the acrosomal region on the head of rat spermatozoa from the caput (Figure 4B), corpus (data not shown), cauda (Figure 4C), and vas deferens (Figure 4D). In contrast, testicular spermatozoa displayed no staining with anti-GPX5 immune serum (Figure 4A), a finding which is consistent with the epididymis being the sole site of GPX5 expression. Preimmune serum (taken from the same rabbit immediately prior to the initial immunization) at the same 1:200 dilution gave a negative result on all rat sperm smears (data not shown).

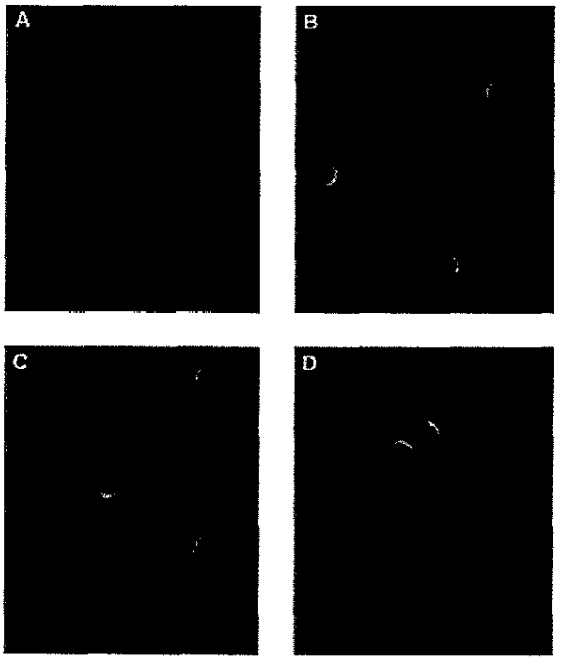

Figure 4. Indirect immunofluorescence localization of glutathione peroxidase isoenzyme (GPX5) on rat spermatozoa using an anti-GPX5 polyclonal antiserum. **(A)** Testicular spermatozoa, **(B)** caput epididymidal spermatozoa, **(C)** cauda epididymidal spermatozoa, **(D)** spermatozoa isolated from the vas deferens. Original magnification: ×630.

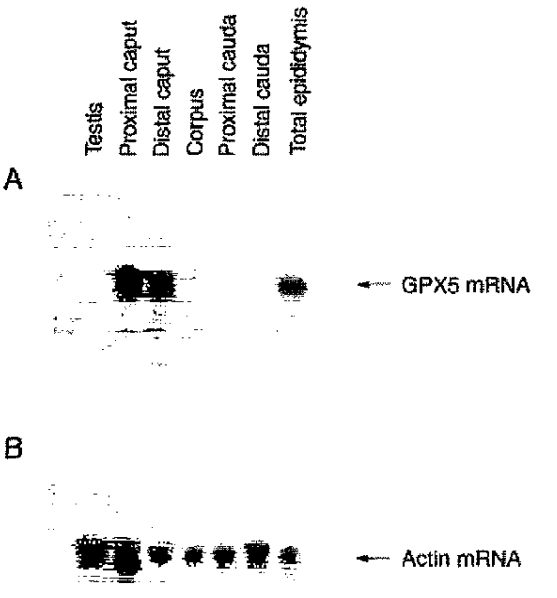

Figure 5. Northern blot analysis of glutathione peroxidase isoenzyme (GPX5) transcripts in the rat epididymis. Total RNA samples (10 μg) isolated from different regions of the rat epididymis, as well as testis, were probed with a rat GPX5 cloned cDNA **(A)**. The blot was subsequently stripped and re-probed with an actin cDNA probe **(B)** to confirm RNA integrity and approximate equivalence of lane loading.

Similar findings have been reported in the mouse where spermatozoa displayed a crescent-like pattern of immunofluorescence characteristic of acrosomal staining, from the mid caput epididymidis and throughout the remainder of the reproductive tract (Jimenez *et al.*, 1990; Vernet *et al.*, 1997).

Region-specific expression of GPX5 transcripts in the rat epididymis

Previous Northern blot analyses had demonstrated the presence of GPX5 transcripts in the epididymis of the rat (Perry *et al.*, 1992), macaque (Perry *et al.*, 1992) and mouse (Ghyselinck *et al.*, 1990; Faure *et al.*, 1991). In the mouse, GPX5 mRNA and protein expression was restricted to the caput epididymidal region (Ghyselinck *et al.*, 1990; Faure *et al.*, 1991).

In order to carry out a more detailed investigation in the rat, total RNA from the proximal caput, distal caput, corpus and cauda regions of adult rat epididymides were subjected to Northern blot analysis and probed with a rat GPX5 cloned cDNA insert (Perry *et al.*, 1992).

After autoradiography for 8 h (Figure 5A), rat GPX5 transcripts of the expected size (1.8 kb; see Perry *et al.*, 1992) were readily detected in total RNA from caput epididymis and total epididymis. A similar high level of GPX5 expression in the rat caput region was also reported by Zini and Schlegel (1997). Much longer exposure of the blot (72 h) in the present study (data not shown) resulted in a faint band appearing with RNA from the corpus and caudal regions, but not in testis RNA. Expression of GPX5 in the human cauda epididymis has also been observed using the highly sensitive technique of RT–PCR (L.Hall and A.F.C.Perry, unpublished data), although the physiological relevance of such low levels, relative to the caput region, is unclear.

The blot was subsequently stripped and re-hybridized with a mouse actin cDNA probe (Humphries *et al.*, 1981) to confirm the integrity of all RNA samples and to check for approximate equivalence of lane loading (Figure 5B).

Developmental expression of GPX5 in the rat epididymis

Total RNA was isolated from rat epididymides removed from juvenile rats at 13, 19, 23, 32 and 44 days post-partum, as well as from adult rats. RT–PCR reactions carried out using rat GPX5-specific oligonucleotides amplified a PCR product of the predicted size (338 bp) with cDNA samples obtained from 32 day and 44 day rat epididymal RNA (Figure 6A). Positive control reactions, using primers specific for GAPDH, were performed in order to confirm the integrity of the synthesized cDNA and correct for differences in RNA, and hence cDNA, yields between the different tissue samples (see Figure 6B).

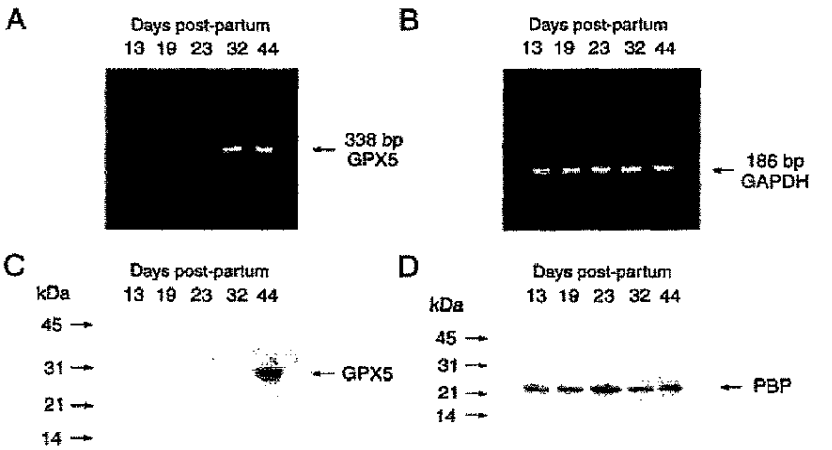

Figure 6. Developmental expression of glutathione peroxidase isoenzyme (GPX5) in the rat epididymis. **(A)** Reverse transcription–polymerase chain reaction (RT–PCR) amplification of GPX5 transcripts using total epididymal RNA obtained from rats of different ages (13, 19, 23, 32 and 44 days post-partum). **(B)** RT–PCR amplification using primers specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm RNA integrity and comparable gel loading. **(C)** Western blot analysis of epididymal homogenates from rats of different ages, probed with anti-GPX5 polyclonal antiserum. **(D)** Western blot analysis of epididymal homogenates probed using an anti-phosphatidylethanolamine-binding-protein (anti-PBP) polyclonal antiserum to confirm equal protein loading.

The TRIzol™ method of tissue extraction results in the retention of protein and DNA in the organic phase with RNA partitioning into the aqueous phase. As a result, the protein constituents can be recovered from the same sample used for RNA preparation. Consequently, approximately equal quantities of protein from each pre-pubertal rat epididymal sample used in the preceding RT–PCR study were resolved by SDS–PAGE. The protein was then transferred to a PVDF membrane and GPX5 detected using the anti-GPX5 polyclonal antiserum.

Rat GPX5 protein was not detected until day 44 post-partum (Figure 6C), corresponding to the time just prior to puberty when the first spermatozoa enter the epididymis. Any apparent delay between the appearance of transcript (clearly visible at day 32) and appearance of the corresponding protein (between day 32 and day 44) is likely to be emphasized by the very high sensitivity of PCR as a detection method compared to Western blotting.

The Western blot was subsequently stripped and reprobed with an antiserum against constitutively expressed rat PBP (Frayne *et al.*, 1998b), to confirm equal lane loading (Figure 6D).

Discussion

Spermatozoa possess defensive mechanisms to prevent ROS damage. Depending on the species, their cytoplasm may contain SOD, GPX/GRD and catalase to counteract internally generated ROS. In rabbit spermatozoa, superoxide anion production and SOD activity are closely matched (Holland *et al.*, 1982); this is essential, as rabbit spermatozoa apparently lack intracellular GPX and catalase. Mice and humans, on the other hand, produce excess superoxide anion, saturating the SOD and necessitating the GPX1/GDP activity (Storey, 1997).

Extracellular superoxide anion can dismutate to hydrogen peroxide and/or singlet oxygen. If unchecked, this hydrogen peroxide can diffuse across the membranes where it can inactivate some of the enzymes responsible for energy production. In addition, singlet oxygen at the membrane surface has sufficient power to induce lipid peroxidation which may result in motility loss and a concomitant reduction in fertilizing capacity.

Glutathione appears to be an important antioxidant in controlling ROS in the epididymis; it is present in high concentrations in the lumen of the epididymis and increases as the epididymis is traversed. It has free radical scavenging properties in its own right but often works as part of a GPX system. Two extracellular GPX isoenzymes have been identified within the epididymis; an epididymis-specific, selenium-independent GPX (GPX5) and plasma GPX (GPX3), both of which may potentially play a role in removing hydrogen peroxide from epididymal fluid. However, whilst the association of GPX5 with the sperm membrane is ideally suited to capturing any hydrogen peroxide traversing the membrane from the epididymal fluid, recent studies on the purified porcine enzyme suggest that GPX5 has very little activity towards hydrogen peroxide or organic hydroperoxides *in vitro* (Okamura *et al.*, 1997). Instead, these authors propose that GPX5, bound to the sperm acrosomal region, may play a role in preventing premature acrosome reaction by binding to lipid peroxides which might otherwise interact with phospholipase A2 and induce the acrosome reaction while spermatozoa are stored within the epididymis.

The expression of rat and mouse GPX5 have been shown to be under tight androgen control (Ghyselinck *et al.*, 1991; Perry *et al.*, 1992; Lefrancois *et al.*, 1993), resulting in total loss of the transcript after castration and almost complete restoration of pre-castration transcript concentrations after subsequent testosterone treatment. Analysis of the mouse

GPX5 genomic sequence and its flanking regions reveal multiple putative androgen response elements, as well as consensus binding sites for the transcription factor PEA-3 (Ghyselinck *et al.*, 1993).

Since epididymal GPX5 associates with spermatozoa its expression might be expected to coincide with the release of spermatozoa into the epididymis. In the rat, we report here that GPX5 transcripts are readily detected at day 32 post-partum and thereafter through to the adult, although GPX5 protein is not clearly detectable until some time after 32 days post-partum; expression being predominantly confined to the proximal caput epididymis. Spermatogenesis begins in the juvenile rat testis ~5 days post-partum, but spermatozoa are not detected within the epididymis until ~44 days. Hence, within the developing epididymis, GPX5 protein is not detectable until about the time the first wave of spermatozoa enter the epididymis.

In the mouse, GPX5 transcripts were first detectable at 10 days post-partum (Lefrancois *et al.*, 1993) with protein synthesis being delayed until day 20. Detection of secreted protein was further delayed until spermatozoa reached the mouse epididymis at day 30 post-partum (Lefrancois *et al.*, 1993). Consequently, in both the rat and the mouse, GPX5 transcripts were readily detectable at earlier times than the corresponding protein, suggesting that post-transcriptional mechanisms may play an important role in regulating the expression and concentration of this protective enzyme.

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